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## **Proteolytic processing regulates placental growth factor activities**

Hoffmann, Daniel C ; Willenborg, Sebastian ; Koch, Manuel ; Zwolanek, Daniela ; Müller, Stefan ;  
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Proteolytic processing regulates Placental growth factor activities\*

Daniel C. Hoffmann<sup>1</sup>, Sebastian Willenborg<sup>1</sup>, Manuel Koch<sup>2,3</sup>, Daniela Zwolanek<sup>2</sup>, Stefan Müller<sup>3</sup>, Ann-Kathrin A. Becker<sup>2</sup>, Stephanie Metzger<sup>4</sup>, Martin Ehrbar<sup>4</sup>, Peter Kurschat<sup>1</sup>, Martin Hellmich<sup>5</sup>, Jeffrey A. Hubbell<sup>6</sup>, Sabine A. Eming<sup>1,3,7</sup>

<sup>1</sup>Department of Dermatology, University of Cologne, Cologne, Germany

<sup>2</sup>Center for Biochemistry, Institute for Dental Research and Musculoskeletal Biology, University of Cologne, Cologne, Germany

<sup>3</sup>Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany

<sup>4</sup>Department of Obstetrics, University Hospital Zurich, Switzerland

<sup>5</sup>Institute of Medical Statistics, Informatics and Epidemiology, University of Cologne, Germany

<sup>6</sup>Institute for Bioengineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

<sup>7</sup>Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany

\*Running title: *Plasmin processing of PlGF*

To whom correspondence should be addressed: Sabine Eming, Department of Dermatology, University of Cologne, Kerpenerstr. 62, 50937 Cologne, Germany, Phone: ++49-221-4783196, Fax: ++49-221-4785949, e-mail: [sabine.eming@uni-koeln.de](mailto:sabine.eming@uni-koeln.de)

**Keywords:** PlGF, angiogenesis, mechanism of growth factor regulation, proteases

**Background:** Mechanisms of Placental growth factor (PlGF)-mediated blood vessel formation are incompletely understood.

**Results:** Plasmin cleaves the heparin binding domain of PlGF-2.

**Conclusion:** Plasmin regulates PlGF-2/Neuropilin-1-mediated tissue vascularization and growth.

**Significance:** Plasmin-mediated carboxyl-terminal processing of VEGF-family members may be considered as a principal mechanism to regulate their biological activity.

## SUMMARY

Placental growth factor (PlGF) is a critical mediator of blood vessel formation, yet mechanisms of its action and regulation are incompletely understood. Here we demonstrate that proteolytic processing regulates the biological activity of PlGF. Specifically, we show that plasmin processing of PlGF-2 yields a protease-resistant core fragment comprising the Vascular endothelial growth factor receptor-1 binding site, but lacking the carboxyl-terminal domain encoding the heparin binding domain (HBD) and an 8-amino acid peptide encoded by exon 7. We have identified plasmin cleavage sites, generated a truncated PlGF118 isoform mimicking plasmin-processed PlGF, and

have explored its biological function in comparison to PlGF-1 and -2. The angiogenic responses induced by the diverse PlGF forms were distinct. Whereas PlGF-2 increased endothelial cell chemotaxis, vascular sprouting and granulation tissue formation upon skin injury, these activities were abrogated following plasmin digestion. Investigation of PlGF/Neuropilin-1 binding and function suggests a critical role for HBD/Neuropilin-1 interaction and its regulation by plasmin processing. Collectively, here we provide new mechanistic insights into the regulation of PlGF-2/Neuropilin-1-mediated tissue vascularization and growth.

Placental growth factor (PlGF), a member of the Vascular endothelial growth factor (VEGF) family of growth factors, is a critical regulator of postnatal angiogenesis in various physiological and pathological conditions including repair of soft <sup>1-3</sup> and hard tissues <sup>4</sup>, inflammation <sup>5</sup>, and cancer <sup>6</sup>. The exact molecular mechanisms by which PlGF regulates blood vessel formation are still not completely understood <sup>7</sup>. PlGF has been reported to support the formation and maturation of new vessels by direct action on existing endothelial cells, but also promotes angiogenesis indirectly by inducing recruitment and survival of other cell types

involved in angiogenesis, such as mural cells, monocytes/macrophages and bone marrow-derived precursor cells<sup>8-10</sup>.

Although PlGF and VEGF-A show only a 42% amino acid sequence identity, as well as significant functional differences, PlGF shares remarkable structural similarities with the extensively analyzed family member VEGF-A<sup>11</sup>. Comparative structure-function analysis between VEGF-A and PlGF might contribute to advance mechanistic insights into PlGF-mediated activities. Generation of diverse VEGF-A isoforms, which are distinguished by the presence of C-terminal peptides encoded by exon 6, 7 and 8 of the *vegfa* gene, are crucial for the diverse biochemical and functional properties of VEGF-A. These include binding to cell surfaces and extracellular matrix (ECM) components, receptor-binding characteristics, endothelial cell adhesion and survival, as well as vascular branch formation<sup>11</sup>. Similarly to the generation of VEGF-A isoforms, in humans mRNA splicing of a single *plgf* gene gives rise to at least 4 different protein variants (PlGF-1 to -4), with PlGF-1 and -2 being the predominant isoforms expressed and studied so far<sup>12</sup>. In mice the *plgf* gene encodes only the PlGF-2 isoform<sup>13</sup>.

PlGF variants are homodimeric molecules, sharing a sequence encoded by exons 1-5 representing the VEGFR-1 binding sites and an 8-amino acid peptide encoded by exon 7. PlGF-1 and -2 differ in the absence or presence of a domain encoded by exon 6, respectively, which exhibits affinity to heparin-sepharose and is referred to as heparin-binding domain (HBD)<sup>14</sup>. Furthermore, cross-linking experiments and competitive binding studies on endothelial cells indicated that the HBD of PlGF-2 is critical for the interaction with the co-receptor neuropilin-1 (Nrp-1)<sup>15,16</sup>. The biological/functional consequences of PlGF-2 binding with Nrp-1 and/or ECM components are still unclear. Likewise, functions of the amino acid sequence encoded by exon 7 have not been reported.

Although mechanistically not yet completely understood, binding of VEGF-A165 through its HBD to Nrp-1 was shown to promote VEGF-A165/VEGFR-2 complex formation and to sustain VEGFR-2 signaling and biological activities<sup>17,18</sup>. Furthermore, differential interaction of VEGF-A isoforms with various ECM components has been

intensively analyzed. All VEGF-A isoforms except the short VEGF-A121 and the VEGF-Axxx forms interact through their HBD encoded by exon 6a and 7 with heparin/heparan sulfate (HS); for VEGF-A165 this feature has been shown to be essential for the establishment of a functional vascular system<sup>19,20</sup>. One could speculate on similar functions for the diverse PlGF isoforms for differential binding to matrix components and/or Nrp-1 interactions.

In addition to differential mRNA splicing, proteolytic processing of long VEGF-A isoforms has been shown to be an important regulatory mechanism to control VEGF-A activities<sup>21</sup>. Specifically, we and others have demonstrated the susceptibility of VEGF-A165 to proteolytic cleavage by plasmin and metalloproteinases<sup>21-25</sup>. Both proteases generate a protease-resistant core fragment containing the VEGFR binding domain, but lacking the carboxyl-terminal domain coding for the HBD and the domain encoded by exon 8. Proteolytic processing of VEGF-A165 substantially reduced and altered the angiogenic response during wound healing and tumor formation<sup>24,25</sup>. Whether the PlGF isoforms display analogous susceptibility to proteases has not been analyzed. Here we investigated the hypothesis that PlGF is a substrate of plasmin and might be regulated in its biological activities by proteolytic processing. We show that PlGF-2 is sensitive to plasmin processing and that truncation of PlGF-2 significantly impacts its HBD/Nrp-1-mediated effects on angiogenesis. Our findings unveil a dual control of PlGF activity by transcriptional regulation and proteolytic mechanisms.

## EXPERIMENTAL PROCEDURES

*Synthesis and purification of recombinant human PlGF variants* - cDNAs of full-length PlGF-1 (Met-1 to Arg-131), PlGF-2 (Met-1 to Arg-152) and a truncated version PlGF118 (Met-1 to Lys-118) were generated from human placenta and cloned into the eukaryotic expression vector pCEP V149 and pCEP V19. For affinity purification cDNAs were fused either to an amino-terminal, 8x histidine tag (PlGF118) or an amino-terminal, 8x histidine tag and carboxyl-terminal, double Strep tag (PlGF-1, PlGF-2). Proteins were produced in human embryonic kidney cells (HEK293 EBNA cells). Recombinant PlGF isoforms were purified to 95% purity by



affinity chromatography using Strep-Tactin Superflow sepharose (PIGF-1 and PIGF-2; IBA BioTAGnology) or immobilized metal ion affinity chromatography using Ni-Sepharose 6 Fast Flow (PIGF118; GE healthcare). Protein identities were confirmed by Peptide Mass Fingerprinting. Protein concentrations were determined by the BCA assay (Pierce) and ELISA (Quantikine human PIGF, R&D Systems) according to the manufacturer's instructions. As indicated, in some experiments recombinant PIGF proteins were purchased and produced in Sf-9 insect cells (Reliatech).

*Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting* - SDS-PAGE was performed following the protocol of Laemmli. To analyze the sensitivity of PIGF isoforms to plasmin, PIGF proteins were incubated with wound exudate, human serum plasmin (0.02 U/mL or serial dilutions as indicated) (Calbiochem), or  $\alpha$ 2-antiplasmin (Calbiochem) at 37°C; at indicated time points, reactions were terminated by the addition of reducing or non-reducing Laemmli buffer and heating to 95°C. The reactions were resolved on a 4-12% reducing Bis-Tris SDS-PAGE gel (NuPAGE, Invitrogen). Integrity of PIGF isoforms was determined by silverstaining (SilverQuest™, Invitrogen) or by detecting immunoreactive products with an anti-human PIGF monoclonal rabbit antibody directed against the first 20 amino-terminal amino acids of human PIGF (Reliatech). Bound primary antibody was detected using an anti-rabbit-HRP conjugated secondary antibody (DAKO A/S). To assess Nrp-1 expression in PAE and PAE/Nrp-1 cells, cell lysates were normalized to 1000  $\mu$ g total protein, incubated with ConA-Sepharose beads (Amersham Biosciences) to enrich glycosylated proteins following the manufacturer's instructions; the eluted fraction was analyzed by immunoblotting using a mouse-anti-human Nrp-1 (A12) antibody (Santa Cruz Biotechnology) and an anti-mouse-HRP conjugated secondary antibody (DAKO A/S).

To analyze signal transduction, endothelial cells were starved and incubated with PIGF variants (2.5 nM) in EBM-2 (growth factor free, 0.1% FCS) (Invitrogen), VEGF-A165 (1.8 nM) (produced in Sf9 insect cells, Reliatech) or starvation medium for indicated time periods. Cells were lysed, samples were resolved in 4-12% Bis-Tris SDS-PAGE gel and immunoblotted with anti-

phospho-focal adhesion kinase (Tyr<sub>576/577</sub>), anti-phospho-Akt (Ser<sub>473</sub>), anti-phospho-Erk-1/-2, (Thr<sub>202</sub>/Tyr<sub>204</sub>), anti-total Akt, anti-total Erk-1/-2, anti- $\beta$ -actin (all Cell Signaling Technologies), anti-phospho-VEGFR-1 (Tyr<sub>1213</sub>) (Millipore), anti-total-VEGFR-1 (Millipore) or anti-Nrp-1 (Santa Cruz Biotechnology). Detection was accomplished using the enhanced chemiluminescence western blot detection system (ECL, Amersham Bioscience). Densitometric analysis of phospho-signal intensity was performed using the ImageJ 1.44p software. The signal of pErk, pAkt or pFAK was normalized to the corresponding total Erk, Akt or  $\beta$ -actin signal, respectively. Multiple Western blots ( $\geq 3$ ) were performed.

*Mass spectrometric analysis* – PIGF-2 expressed in HEK293 cells (25  $\mu$ g in 200  $\mu$ L 50 mM Tris/HCl, pH 8) was incubated with Ni-sepharose beads (15 min, RT) to allow binding to the N-terminal his-tag, followed by incubation in plasmin (final concentrations: 0.04 or 0.008 U/mL in plasmin buffer 50 mM Tris/HCl, pH 8, 37°C, 5 or 30 min); beads were collected by centrifugation (5 min, 13,500 rpm, 4°C), and the supernatants were analyzed by LC-MS/MS. Liquid chromatography (LC)-MS data were acquired on a Q-ToFII quadrupole-TOF mass spectrometer (Micromass) equipped with a Z spray source. Samples were introduced by an Ultimate Nano-LC system (LC Packings) equipped with the Famos autosampler and the Switchos column-switching module. The column setup comprises a 0.3-mm-by-1-mm trapping column and a 0.075-by-150-mm analytical column, both packed with 3  $\mu$ m Atlantis dC18 (Waters). Samples were diluted 1:10 in 0.1% TFA. A total of 10  $\mu$ L was injected onto the trap column and desalted for 1 min with 0.1% TFA and a flow rate of 10  $\mu$ L/min. The 10 port valve switched the trap column into the analytical flowpath, and peptides were eluted onto the analytical column by using a gradient of 2% acetonitrile (ACN) in 0.1% FA to 40% ACN in 0.1% FA over 65 min and a column flow rate of ca. 200 nL/min, resulting from a 1:1,000 split of the 200  $\mu$ L/min flow delivered by the pump. The electrospray ionization (ESI) interface comprised an uncoated 10  $\mu$ m i.d PicoTip spray emitter (New Objective) linked to the HPLC flowpath using a 7  $\mu$ L dead volume stainless steel union mounted onto the PicoTip holder assembly (New Objective). Stable nanospray was established by the

application of 1.7 to 2.4 kV to the stainless steel union. The data-dependent acquisition of MS and tandem MS (MS/MS) spectra was controlled by the Masslynx 4.0. Survey scans of 1.4 s covered the range from  $m/z$  400 to 1,400. Doubly and triply charged ions rising above a given threshold were selected for MS/MS experiments. In MS/MS mode, the mass range from  $m/z$  40 to 1,400 was scanned in 1.4 s, and 4 scans were added up for each experiment. Micromass-formatted peak lists were generated from the raw data by using the Proteinlynx software module. A database search using a local installation of MASCOT 1.9 and a custom database containing the sequence of recombinant PlGF-2 was used for a fast identification of PlGF-2 derived peptides. No enzyme specificity was used for the database search. Since it was expected, that the sequence stretch of interest contains a pair of oxidised cysteines, cysteine oxidation was allowed as optional modification. Results reported by the search engine were verified by manual inspection of the deconvoluted spectra.

**Surface Plasmon Resonance spectroscopy** - Binding experiments were performed by surface plasmon resonance measurements on a Biacore 3000 instrument (Biacore Inc.) at 25°C, following a well established procedures<sup>26</sup>. Briefly, to assess the binding capacity of the various PlGF isoforms to Nrp-1 in dependence to heparin, a CM5 sensor surface was activated by use of EDC/NHS and coupling buffer in a ratio of 1:1 as activating reagents. The degree of coupling was set to approximately 1500 RU. Remaining reactive groups were inactivated with ethanolamine, and PlGF variants were coupled on chip surface at a flow rate of 5 µL/min. The following binding experiments with human Nrp-1 (extracellular domain, Phe22-Lys644; BD Biosciences) as soluble analyte were performed with various concentrations (1 to 300 nM in HEPES running buffer). The analyte was passed over the sensor chip with a constant flow rate of 30 µL/min for 300 seconds, dissociation was measured over 500 seconds. Between different experimental cycles, the bound proteins were washed from the sensor surface with 2 M NaCl in running buffer. In a second experimental setting, Nrp-1 was coupled to the sensor chip and the degree of coupling was set to 1500 RU, whereas 1 to 300 nM of PlGF was used as soluble analyte. Fittings of the data, overlay plots and calculation of  $K_D$ -values were done

with BIAevaluation software 4.1 estimating a 1:1 model.

**Binding of PlGF forms to heparin** - The three proteins were dialyzed against the loading buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.4), and 1 mg each was applied onto a heparin-Sepharose CL-6B column (GE healthcare). After five washes with loading buffer, the columns were eluted stepwise with increasing salt concentration (0.1, 0.2, 0.4, 0.5, 0.75, 1 and 2 M NaCl in 20 mM Tris-HCl, pH 7.4) and the samples were analyzed by SDS-PAGE.

**Circular dichroism (CD) spectroscopy** - CD spectra (180-260 nm) were recorded at 100 nm/min using a Jasco-J715 spectropolarimeter and a thermostated 0.1 cm path length quartz cell (20°C, 5 accumulations each). Data point resolution and bandwidth were set to 0.2 nm, sensitivity to 50 mdeg. CD spectra of samples containing 5 µM PlGF (PlGF-1, PlGF-2 or PlGF118) in PBS were measured, background-corrected, converted to mean molar residue ellipticity and data were evaluated using J-715 for Windows Standard Analysis software.

**Chemotaxis assay** - Chemotaxis assays were performed using a Boyden chamber system with filter inserts of 8 µm pore size (Millipore). Filters were pre-coated with collagen (BD). Medium supplemented with or without PlGF isoforms (2.56 nM) was added into the lower chamber, whereas  $2.5 \times 10^5$  cells were seeded into the upper chamber. Medium supplemented with 10% FCS or VEGF-A165 (1.79 nM) (Reliatech) served as positive control. After 4.5 hours (37 °C) cells on the lower face were fixed and stained with a variant of Romanowski staining (Quickdiff, Behring) and analyzed by light-microscopy; cell number was determined in 10 high power fields per filter (200-fold magnification).

**3D Spheroid-sprouting Assay** - HUVE cells (1000 cells/well) were seeded in 20% methylcellulose/80% growth medium (100 µL) into a 96-roundbottom plate (NUNC) to induce spheroid formation. After 24 hours of incubation (37°C), spheroids were harvested and 50 spheroids/gel were seeded into 500 µL of a non-polymerized collagen gel (2 mg/mL) in 24-well plates. Following polymerization, PlGF variants (3.66 nM) or VEGF-A165 (1 nM) as positive control were added to the gel. After 24 hours incubation at 37°C, 15 randomly chosen spheroids were analyzed for each protein at 100-fold magnification (Nikon

bright field microscope). Cumulative sprouting length and number of sprouts/spheroid was measured using the ImageJ software.

*Animals and wounding experiments* - C57BLKS/J-m+/+Lepr<sup>db/db</sup> mice (Jackson Laboratory, male, 10-12 weeks of age) were caged individually under standard pathogen-free conditions and two independent wound healing experiments were performed as previously described<sup>25</sup>. Briefly, mice were anesthetized, 4 full-thickness punch-biopsy wounds were created on the back of the mouse and treated locally for the following 6 days by either applying PIGF variants (2.5  $\mu$ M in PBS) or vehicle control (PBS). Solution was allowed to adsorb for at least 1 hour before the animal was placed back into its cage. For histological analysis wound tissues were excised and the wound area bisected in caudocranial direction; the tissue was fixed (4% paraformaldehyde) or embedded in OCT compound (Tissue Tek), immediately frozen in liquid nitrogen, and stored at -80°C. Histological analysis was performed on serial sections (6  $\mu$ m).

*Histology, immunohistochemistry and morphometric analysis* - Cryosections were fixed in acetone, blocked (3% BSA), incubated with antisera against murine CD31 (BD Biosciences), desmin (DakoCytomation), and F4/80 (Dianova); bound primary antibodies were detected using an ALEXA Fluor 488-conjugated polyclonal goat anti-rat antibody and an ALEXA Fluor 594-conjugated polyclonal goat anti-mouse antibody (Invitrogen); nuclei were stained with DAPI. As a control for specificity primary antibodies were omitted and replaced by irrelevant isotype-matched antibodies. Immunofluorescence/immunohistochemical microscopy was conducted at indicated magnifications (Microscope Eclipse 800E; Nikon), and morphometric analysis was performed on digital images as recently described<sup>25</sup>.

*Human wound exudates* - Wound exudate was obtained from patients presenting with non-healing skin ulcers due to venous insufficiency (n=7) or from patients with normally healing acute cutaneous wounds at indicated time points post injury (day 2 n=10, day 7 n=5, day 14 n=7; excisional wounds of the lower leg awaiting wound closure by secondary intention); in addition blood serum was obtained from 15 patients. Exudate was

harvested and tested for plasmin activity as previously described<sup>23,26</sup>. PIGF content in serum samples or wound exudates was determined by ELISA (Quantikine human PIGF immunoassay, R&D Systems).

*Statistical analysis* - Data were summarized by mean  $\pm$  SEM. Location differences were assessed by analysis of variance and Tukey post-hoc tests for pairwise comparisons. Thus, the type I error is strongly controlled, at least pertaining to specific families of (pairwise) hypotheses. Moreover, in case the SEM clearly increased with the mean (based on Bartlett's test for equal variances), a log-transformation was applied in order to stabilize the variance. Statistical significance (adjusted for multiplicity) was graded according to \* < 0.05, \*\* < 0.01, or \*\*\* < 0.001. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc.).

## RESULTS

*PIGF is cleaved by plasmin* - To investigate the hypothesis that PIGF might be a substrate for the serine protease plasmin, PIGF-1 and PIGF-2 were incubated with plasmin and samples were subjected to gel-electrophoresis and analyzed by silver-staining (Fig. 1A-C) and Western blotting (Fig. 1D). In non-reducing conditions dimeric PIGF-2 migrated with a molecular weight of approximately 42 kDa (Fig. 1A). Extended incubation of PIGF-2 with plasmin for 4 hours resulted in its fragmentation and formation of a protease-resistant cleavage product of approximately 32 kDa. In reducing conditions, the PIGF-2 monomer exhibited an electrophoretic mobility shift from 23 kDa to approximately 16 kDa following plasmin-mediated digestion (Fig. 1B). Cleavage products migrating below 16 kDa could not be detected. Plasmin-mediated fragmentation of PIGF-2 was inhibited by the plasmin inhibitor  $\alpha$ 2-antiplasmin (Fig. 1B). In reducing conditions, monomeric PIGF-1 migrated with a molecular weight of 17 kDa, with a shift of approximately 1-2 kDa following plasmin digestion (Fig. 1C). The generation of a major protease-resistant fragment with similar electrophoretic mobility for PIGF-1 and -2 as well as the detection of this protease-resistant 16 kDa fragment with an antibody raised against the amino-terminal domain of PIGF, suggests carboxyl-terminal cleavage in both proteins (Fig. 1D). A dose-response analysis

indicated that plasmin-mediated cleavage of PIGF-2 occurred in multiple stages (Fig. 1D).

To examine whether PIGF proteolysis might be a biologically relevant event in the human system, we determined the concentration and integrity of PIGF in exudates obtained from normal healing or non-healing chronic human wounds. Wound exudate is the interstitial fluid of injured tissue that reflects the metabolic condition of the wound microenvironment and has been proven to be useful for identification of factors involved in wound healing physiology and pathology<sup>27,28</sup>. As quantified by ELISA, in both exudates obtained from healing and non-healing wounds the mean concentration of PIGF was significantly increased (at least 25-fold) as compared to the mean concentration of plasma serum levels of 26 pg/mL, arguing for local PIGF expression at the wound site (Fig. 1E). PIGF concentrations were similar in healing and non-healing wounds. To analyze the integrity of PIGF protein in the wound environment, recombinant PIGF-2 was spiked into wound exudates obtained from normal healing or non-healing wounds and samples were subjected to Western blot analysis in reducing conditions (Fig. 1F). Whereas in exudates obtained from healing wounds to large extent PIGF-2 maintained its stability, in exudates obtained from non-healing wounds PIGF-2 underwent a gradual degradation resulting in the formation of a protease-resistant fragment with an approximate molecular weight of 16 kDa after 2 hours of incubation with exact the same electrophoretic mobility than plasmin digested PIGF-2 (Fig. 1F). Fragmentation of PIGF-2 (produced in human embryonic kidney 293 EBNA cells) in exudates obtained from non-healing wounds could be partially rescued by pre-incubation with  $\alpha$ 2-antiplasmin (Fig. 1G). As shown previously<sup>27,28</sup>, in this study plasmin activity was significantly increased in exudates obtained from chronic versus healing wounds [plasmin activity in chronic exudates (n=3) mean 27 mU/mL  $\pm$ SD 7.8, in healing exudates (n=4) mean 17 U/mL  $\pm$ SD 5.7;  $p = 0.05$ ]. Collectively, these findings suggest that proteolytic processing of the carboxyl-terminus in PIGF occurs during tissue repair and is differentially regulated in healing and non-healing conditions.

*Processing of PIGF-2 by plasmin results in cleavage of the HBD* - To identify the plasmin cleavage sites in PIGF-2 we used

LC-MS/MS analysis. Consistent with the Western blot analysis that suggested a multi-stage cleavage event, LC-MS/MS analysis revealed several fragments that indicate cleavage sites within the HBD at residues Arg151, Lys136, Arg134, Lys131, Lys127, and Lys118 (Fig. 2). Extended plasmin digestion therefore results in a complete degradation of the HBD, with Lys118 representing position P1 of the cleavage site closest to the amino-terminus, and the plasmin-resistant amino-terminal fragment PIGF1-118 representing the VEGFR-1-binding domain. Although we did not subject plasmin-digested PIGF-1 fragments to LC-MS/MS analysis, several arguments suggest that also in PIGF-1 the position Lys118 is the plasmin cleavage site closest to the amino-terminus: first, the cleavage site Lys118-Met119 is localized within the domain encoded by exon 5, which is equivalent in PIGF-1 and -2; secondly, cleavage at position Lys118 would be consistent with the electrophoretic mobility shift of 1-2 kDa under reducing conditions following plasmin processing of PIGF-1 (Fig. 1C). Notably, the plasmin cleavage site for VEGF-A165 has also been mapped within exon 5 (Fig. 2A)<sup>22,23</sup>.

*Plasmin-cleavage attenuates PIGF-2-mediated VEGFR-1 phosphorylation* - To evaluate the functional impact of plasmin-mediated carboxyl-terminal processing of PIGF, we generated constructs of PIGF-1, PIGF-2 and a truncated isoform that mimicked the plasmin-resistant PIGF fragment (PIGF118). These constructs were used to produce recombinant proteins in human embryonic kidney (HEK) 293 EBNA cells. To assess proper folding of the PIGF-variants, we analyzed dimerization and secondary structure properties by SDS-PAGE analysis and CD-spectroscopy. In non-reducing conditions, PIGF-2, PIGF-1 and PIGF118 migrated with a molecular weight of approximately 65-68 kDa, 58-60 kDa, and 54-55 kDa, respectively (Fig. 3A). In reducing conditions, monomers of PIGF-2, PIGF-1 and PIGF118 exhibited a molecular weight of approximately 38-39 kDa, 36-37 kDa, and 32-33 kDa, respectively (Fig. 3A). Differences in electrophoretic mobility of recombinant proteins depicted in figure 1 and 3 are due to differences in posttranslational modifications (e.g. glycosylation) and histidine/double Strep-tag expression. All isoforms showed a comparable degree in their ellipticity, indicating that loss of the carboxyl-



terminus does not affect the secondary structure of PlGF isoforms (Fig. 3B). As revealed by Western blot analyses, all isoforms resulted in Tyr<sub>1213</sub> phosphorylation of VEGFR-1 when compared to stimulation with 0.1% FCS, although weaker in PlGF-1 and PlGF118 when compared to PlGF-2 (Fig. 3C).

*Plasmin-cleavage attenuates PlGF-2-mediated endothelial cell chemotaxis and vascular sprouting* - To analyze the functional impact of plasmin-processing for PlGF, the chemotactic activity of PlGF variants on endothelial cells was analyzed in a Boyden chamber assay. Previous studies reported on equal chemotactic potency of PlGF-1 and -2 (produced in Sf9 insect cells) in bovine aortic arch-derived endothelial cells<sup>16</sup>. Here we used porcine aortic endothelial cells (PAE) stably transfected with the human Nrp-1 receptor (PAE/Nrp-1) or untransfected cells lacking Nrp-1 expression (Fig. 4A) to examine the effect of diverse PlGF variants. Nrp-1 transfected PAE cells treated with PlGF-2 (expressed in Sf9 insect cells) exhibited at least a 2-fold higher chemotactic response as compared to cells treated with PlGF-1 or control cells (Fig. 4B). Pre-incubation of PlGF-2 with plasmin resulted in a significant loss of its chemotactic activity that was partly rescued by  $\alpha$ 2-antiplasmin. Both PlGF-1 and PlGF-2 were unable to induce chemotaxis in untransfected cells lacking Nrp-1 expression (Fig. 4B). Repeating the experiment with PlGF isoforms produced in HEK293 cells and extending the analysis on HUVE cells substantiated the critical role of the HBD for endothelial cell chemotaxis. In both PAE/Nrp-1 and HUVE cells, chemotactic activity was significantly increased in response to PlGF-2 as compared to PlGF-1 and PlGF118 (Fig. 4C, D).

We next tested the role of plasmin processing of PlGF in endothelial sprout formation using a 3D-spheroid outgrowth assay in type collagen I gels. Both the cumulative sprouting length (reflecting sprout outgrowth) and the number of sprouts (reflecting sprout induction) per spheroid were significantly increased by PlGF-2 treated cultures as compared to controls (Fig. 4E, F). Although PlGF-1 and PlGF118 also revealed stimulating activity on sprout formation when compared to controls, the overall response was weaker when compared to PlGF-2 and the response on sprout induction did not reach statistical significance when compared to

controls. Of note, after 24 hours of treatment, all PlGF-treated spheroids revealed a greater stability as compared to control cultures, suggesting that PlGF acts as a survival factor for endothelial cells, independently of the HBD and/or the carboxyl-terminal domain encoded by exon 7.

*The HBD of PlGF-2 is critical for its repair promoting activities in diabetic mice* - Further exploration of biological effects of plasmin processing of PlGF was performed in an *in vivo* wound healing assay. Vascular growth is profoundly involved in effective tissue repair and has been shown to be significantly attenuated in preclinical models of impaired healing such as diabetic mice and chronic skin ulcers in humans<sup>23,25</sup>. Full thickness excisional skin wounds were created on the back of diabetic mice (db/db mice) and the repair response was characterized following local treatment with repetitive applications of PlGF isoforms (2.5  $\mu$ M) or vehicle control. Quantitative analysis of granulation tissue formation (H&E stained wound sections), macrophages (F4/80 positive cells), vascular density and perivascular cells (CD31/desmin double staining) present in granulation tissue showed that in PlGF-2 treated wounds the amount (Fig. 5), cellularity and vascularization of granulation tissue (Fig. 6) were significantly increased at day 10 and 14 post injury, as compared to PlGF-1, PlGF118 or vehicle treated wounds. Granulation tissue formation and vascularization were also increased in response to PlGF-1 and PlGF118 as compared to vehicle controls, however these effects were minor and did not reach statistical significance. Furthermore, whereas in PlGF-1- and PlGF118-treated wounds the vascular density decreased towards day 14 post injury, the increased vascular density in PlGF-2-treated wounds sustained until day 14. The ratio of desmin/CD31-double stained vascular structures within the granulation tissue was significantly increased in response to PlGF-2 as compared to PlGF-1-, PlGF118- and vehicle-treated wounds at day 10 and 14. These findings indicate an enhanced coverage of vascular structures by perivascular cells in PlGF-2-treated wounds and suggest increased stability/maturation of PlGF-2-induced blood vessels at the wound site (Fig. 6B). Wound closure kinetics was not significantly altered in PlGF treated wounds at day 10 and 14 post injury (data not shown).

*Plasmin processing of PlGF-2 abrogates its binding capacity to heparin and Neuropilin-1* - Next we evaluated the impact of plasmin processing upon the binding capacity of PlGF to heparin and to the co-receptor Nrp-1. As shown by affinity purification PlGF-2 binds strongly to heparin (Fig. 7A), confirming earlier studies that reported binding of PlGF-2 to heparin-sepharose<sup>14</sup>. Importantly, PlGF-1 and PlGF118 do not interact with heparin (Fig. 7A). As revealed by plasmon resonance spectroscopy, binding of PlGF-2 to the immobilized Nrp-1 extracellular domain was clearly concentration dependent and the calculated  $K_D$  was 0.1  $\mu$ M (Fig. 7B). Plasmin digestion of PlGF-2 abrogated interaction with Nrp-1. PlGF-1 did not bind to Nrp-1. Binding characteristics for Nrp-1 were confirmed for all PlGF variants by inverted settings using Nrp-1 as soluble analyte (data not shown).

*The HBD differentially regulates PlGF-2-induced phosphorylation of the focal adhesion kinase but does not modulate Erk-1/-2 or Akt-mediated signaling* - We further explored downstream pathways that might mediate the differential effects in response to plasmin processed PlGF-2. We focused the analysis on signaling molecules previously shown to be critically involved in PlGF-mediated cell functions. Stimulation of HUVE cells with either PlGF-2 or PlGF118 resulted in rapid phosphorylation of Erk-1/-2 and Akt, which was similar in signal intensity for both molecules (Fig. 8A). Also, overexpression of Nrp-1 in PAE cells did not alter the signal strength for Erk-1/-2 and Akt activation in response to PlGF-2 (Fig. 8B). Based on these findings it seems unlikely that Erk/Akt activation is involved in differential endothelial cell activities in response to PlGF-2 and PlGF118 stimulation. We then investigated the involvement of focal adhesion kinase (FAK) signaling in response to PlGF stimulation. Whereas stimulation with PlGF-2 provoked an increased and sustained phosphorylation signal of FAK, the activation signal was substantially weaker in response to PlGF118 (Fig. 8C). Furthermore, PlGF-2-mediated activation of FAK was pronounced in PAE-Nrp-1 transfected cells as compared to cells lacking Nrp-1. Together, our findings propose a critical role of PlGF-2/Nrp-1 interaction for FAK activation.

## DISCUSSION

In this study we demonstrated that PlGF-1 and -2 are substrates for plasmin and that plasmin-catalyzed cleavage results in loss of the carboxyl-terminal domain in both proteins. Plasmin-mediated processing of PlGF-2 significantly decreased its binding to heparin and Nrp-1, and substantially attenuated its pro-angiogenic activities. Our findings provide novel mechanistic insights into the regulation of PlGF-2/Nrp-1 interactions by proteolytic processing and important biological consequences of this event for tissue vascularization and growth.

We and other investigators previously demonstrated the critical role of plasmin processing of VEGF-A165 for its biological behavior<sup>21,22,25</sup>. In addition, plasmin-mediated processing has been reported to be critical for VEGF-C and -D activation, which are primarily involved in lymphangiogenesis<sup>29</sup>. Here we show that kinetics and structural consequences of plasmin-mediated processing of PlGF-2 are similar to plasmin-mediated cleavage of VEGF-A165. Both proteins reveal gradual degradation following exposure to plasmin, with the highest proteolytic sensitivity within their HBD localized at the carboxyl-terminus. In both proteins the cleavage site closest to the amino-terminus is localized within the domain encoded by exon 5, in close proximity to the cysteine residues critical for the formation of intrachain disulfide bonds. Thus, for VEGF-A165 and PlGF-2 plasmin-mediated cleavage yields an amino-terminal homodimeric VEGF-A110 and PlGF118, respectively, comprising the VEGFR binding domain and lacking the carboxyl-terminal domains. Plasmin processing apparently does not substantially affect folding and dimerization of either protease-resistant fragment, which are still capable of activating VEGFRs although at different levels. Therefore, we propose to consider the carboxyl-terminal processing of these two VEGF-family members by plasmin as a principal mechanism to regulate their biological activity.

Plasmin processing revealed different functional consequences for PlGF-1 and -2. Whereas plasmin digestion of PlGF-2 significantly attenuated endothelial cell chemotaxis, vascular sprouting and granulation tissue formation during tissue repair, biological responses to PlGF-1 were similar to PlGF118 or vehicle control stimulated cells/tissues.

Therefore, effects of plasmin digestion on the biological activities of PlGF-1 appear to be minimal. These findings strongly indicate that most of the PlGF-2-stimulated activities we observed in our studies are dependent upon the HBD encoded by exon 6.

To examine the molecular mechanisms underlying the loss of PlGF-2-mediated responses following plasmin digestion, we investigated several potential mechanisms. First, we assessed whether endothelial cell stimulation with different PlGF variants leads to differences in VEGFR-1 tyrosine phosphorylation and activation of known downstream mediators. PlGF is capable of signaling directly through VEGFR-1, and several VEGFR-1 tyrosine phosphorylation sites in endothelial cells have been reported to be activated upon stimulation with PlGF-2<sup>8,30</sup>. However, we could not find a systematic analysis in the literature investigating the pattern and function of different tyrosine phosphorylation sites of VEGFR-1 in response to diverse PlGF isoforms. Here we show that following stimulation of HUVE cells, all PlGF variants, although weaker in the absence of sequences encoded by exon 6 and/or 7, had the potency to phosphorylate Tyr<sub>1213</sub> and to equally activate downstream mediators including Erk and Akt. Therefore, this pathway is unlikely to explain the different biological activities we found in response to various PlGF isoforms. But these results on downstream signaling support the observation that all PlGF variants mediated stability of spheroid structures when compared to vehicle control, and indicate that PlGF-mediated endothelial cell survival is independent of the HBD and/or the carboxyl-terminal domain encoded by exon 7 as well as binding to Nrp-1 and/or heparin. Furthermore, our findings suggest that PlGF-2-mediated activation of Erk-1/-2 is independent of Nrp-1 binding, which is paralleled by recent reports demonstrating that VEGF-A165-mediated activation of Erk-1/-2 is independent of Nrp-1<sup>31</sup>. At this stage we cannot exclude that other VEGFR-1 tyrosine residues than Tyr<sub>1213</sub> might be differentially activated by different PlGF variants and this aspect requires more detailed examination in future studies.

Secondly, we investigated functional consequences of differential binding of PlGF variants to the co-receptor Nrp-1. The exact mechanism how Nrp-1 acts on endothelial cell functions is still not resolved and so far most

of the functional studies were performed with VEGF-A165<sup>32,33</sup>. Based on those studies it was proposed that Nrp-1 acts as a co-receptor for VEGF-A165, enhancing VEGF binding to VEGFR-2, and thus increasing VEGFR signaling. More recent studies indicated that rather than affecting VEGFR-2 activity directly, Nrp-1 is likely to modulate signaling kinetics and the activation of specific signal output via VEGFR-2<sup>17</sup>. The short carboxyl-terminal sequence of VEGF-A encoded by exon 8a (KPRR) was identified as a critical sequence to direct binding and specificity towards Nrp-1<sup>17,34-37</sup>. Interestingly, against common knowledge, recently it was shown that also VEGF-A121 binds directly to Nrp-1<sup>38</sup> through exon 8a<sup>39</sup>. In contrast to the situation with VEGF-A165, VEGF-A121 was not able to bridge VEGFR-2/Nrp-1 interaction and to induce the formation of a VEGFR-2/Nrp-1 complex<sup>38</sup>. The understanding for PlGF/Nrp-1 interaction is so far based on earlier cross-linking experiments and competitive binding analysis on endothelial cells that identified the HBD of PlGF-2 as an epitope for the Nrp-1b1b2 domain, whereas PlGF-1 revealed no binding to Nrp-1<sup>15,16</sup>. Interestingly, the short carboxyl-terminal sequence of PlGF-1 and -2 encoded by exon 7 has high sequence homology with exon 8a of VEGF-A. In light of the critical role of exon 8a of the *vegfa* gene for Nrp-1 binding<sup>17,34-37</sup>, we hypothesized whether similarly to VEGF-A121, also PlGF-1 might be able to bind to Nrp-1. Using surface plasmon resonance spectroscopy, here we demonstrated that PlGF-2 but not PlGF-1 specifically and strongly binds to Nrp-1. Furthermore, we show that plasmin processing of PlGF-2 completely abrogates binding of PlGF-2 to Nrp-1. Thereby, our findings support earlier cross-linking experiments and corroborate the critical role of exon 6 and not exon 7 as Nrp-1 binding sequence in PlGF-2. Given the sequence homology between the basic peptides encoded by exon 8a and exon 7 of *vegfa* and *plgf*, respectively, we believe this is an interesting finding unraveling a potential difference in binding of PlGF and VEGF-A to Nrp-1, and merits further investigation in future studies.

Our findings indicate a critical role of PlGF-2/Nrp-1 binding for PlGF-2 function. FAK activation is closely linked to cell survival and migration in endothelial cells<sup>40</sup>. Interestingly, a recent report demonstrated the

critical role of VEGF-A165 binding to Nrp-1 for FAK activation, endothelial cell migration and tube formation<sup>31</sup>. We hypothesized that increased FAK activation in response to PlGF-2/Nrp-1 stimulation mediates the findings of superior PlGF-2 pro-angiogenic activities over those of PlGF-1 or PlGF118. Here we substantiated this hypothesis by showing, that phosphorylation of FAK was increased and sustained by PlGF-2 when compared to PlGF118. Furthermore, this effect was enhanced in Nrp-1-transfected PAE cells when compared to cells lacking Nrp-1 expression. Thus, our findings indicate an important role for PlGF-2 binding to Nrp-1 in pathways controlling actin cytoskeletal rearrangements critical for cell migration and vessel growth.

Third, differential interaction of VEGF-A isoforms with diverse components of the ECM, in particular glycosaminoglycans, has been shown to be critical for VEGF-A-mediated biological activities<sup>21</sup>. Here we provide evidence that PlGF-2, but neither PlGF-1 nor PlGF118 binds strongly to heparin and that this interaction is entirely abrogated by plasmin processing. These findings substantiate earlier studies demonstrating high affinity of PlGF-2 to heparin-sepharose<sup>14</sup>. At this stage we can only speculate on potential functions of PlGF-2/heparin binding for angiogenic processes. As reported for VEGF-A165, PlGF-2/heparin interactions might allow for the formation of growth factor gradients which are critical for vascular sprouting and/or modulate Nrp-1-mediated activities<sup>40,41</sup>.

Finally, our findings raise the general question on the biological role of PlGF-1 per se, and on the dual generation of a short PlGF isoform lacking the HBD by either transcriptional control and/or proteolysis. Contrarily to the generation of diverse PlGF isoforms by differential mRNA splicing in humans, in mice PlGF-2 is the only isoform identified so far<sup>13</sup>. Hence, our findings suggest that in mice modification of PlGF-2 activity by proteolytic processing provides a unique mechanism to control activities mediated by the carboxyl-terminal domain. In humans, next to mRNA splicing of the *plgf* gene, proteolytic processing of PlGF isoforms provides an additional mechanism to control processes mediated by the carboxyl-terminal domain. Our findings indicate that the impact of proteolytic processing, specifically on PlGF-2 becomes particularly critical in conditions of high/uncontrolled proteolytic

activity, as e.g. in degenerative conditions such as chronic wounds associated with diabetes mellitus, age and vascular disease. In fact, here we showed that proteolytic processing of PlGF is increased in human chronic venous ulcers when compared to healing wounds. As previously shown for VEGF-A165<sup>25</sup>, this process might contribute to attenuated angiogenesis and tissue growth, which is a hallmark of chronic skin ulcers, the most common cause of impaired healing conditions in humans.

The findings reported here might be of relevance for the development of PlGF-based concepts of therapeutic vascular growth in the field of regenerative medicine. Current molecular/cellular based therapeutic angiogenesis trials showed only limited beneficial effects for some patients with ischemic diseases and there is a need for the development of alternative treatment strategies<sup>42</sup>. In pre-clinical studies PlGF has been shown to be a potent inducer of tissue vascularization<sup>1,3,9,43</sup> often without the side effects reported for VEGF-A therapy. Therefore, PlGF alone or in combination with other pro-angiogenic factors has been proposed for angiogenic therapies. Based on our findings we propose the use of the PlGF-2 isoform due to its superior activity, when compared to PlGF-1. Moreover, in conditions of unrestrained inflammation with unbalanced proteases we suggest to protect PlGF-2 from proteolytic processing to maintain its integrity and biological activity.



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## FOOTNOTES

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### Authors' contributions

D.C.H. and S.A.E. designed and performed experiments, analyzed data and wrote the manuscript.; M.K. and D.Z. performed and analyzed the surface plasmon resonance spectroscopy; S.Mü. performed and analyzed the mass spectrometric analysis; A.A.B. performed and analyzed the CD spectroscopy; M.H. performed the statistical analysis; S.W., S.Me., M.E., P.K. and J.A.H. designed experiments and helped to write the manuscript.

### Abbreviations

CD, circular dichroism; EBM-2, endothelial basal medium-2; ECL, enhanced chemiluminescence; ECM, extracellular matrix; FAK, focal adhesion kinase; HBD, heparin binding domain; HEK cells, human embryonic kidney cells; HUVE cells, human umbilical vein endothelial cells; LC-MS/MS, liquid chromatography tandem mass spectrometry; Nrp-1, neuropilin-1; PAE cells, porcine aortic endothelial cells; PlGF-1/-2, placental growth factor-1/-2; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

### Disclosures

The authors have no conflict of interest to declare.



## FIGURE LEGENDS

**FIGURE 1. PlGF is cleaved by plasmin.** (A–D) PlGF-1 and -2 were incubated with plasmin and  $\alpha$ 2-antiplasmin ( $\alpha$ 2-AP) for different time periods, subjected to non-reducing or reducing gel-electrophoresis and analyzed by (A–C) silver-staining and (D) Western blotting. (E) Quantification (ELISA) of endogenous PlGF protein in exudates obtained from healing or non-healing human wounds and blood serum; each dot represents a sample of a different patient. (F) Representative Western blot analysis of PlGF-2 incubated without/with plasmin or spiked into wound exudates. (G) Shown is a representative Western blot analysis of PlGF-2 incubated without/with plasmin or spiked into wound exudates pre-incubated without/with  $\alpha$  2-AP and the mean fraction of intact/fragmented PlGF-2  $\pm$ SEM of multiple blots (n=3). (A–F: PlGF proteins were expressed in Sf9 cells; G: PlGF-2 protein was expressed in HEK293 cells).

**FIGURE 2. Identification of plasmin cleavage sites in PlGF-2 by LC-MS/MS analysis.** PlGF-2 (expressed in HEK293 cells) was incubated with plasmin and peptide fragments were analyzed by LC-MS/MS. (A) Schematic presentation of plasmin cleavage sites (arrowheads) in PlGF-2 based on identified fragments; the HBD in PlGF-2 and VEGF-A165 is illustrated in grey; alignment of plasmin cleavage sites closest to the amino-terminus of VEGFA165, PlGF-1 and -2. (B) Absolute and relative deviation between expected and found molecular weight of PlGF-2 peptide fragments identified by LC-MS/MS.

**FIGURE 3. PlGF-isoforms expressed in HEK293 cells are biologically active.** (A) SDS-PAGE and Coomassie stain, and (B) CD-spectroscopy of PlGF-isoforms expressed in HEK293 cells. (C) Stimulation of HUVE cells with PlGF-isoforms (2.5 nM) and analysis of VEGFR-1 phosphorylation (Tyr<sub>1213</sub>); the degree of phosphorylation was determined by densitometric analysis of phospho-signal intensity and normalized to the corresponding  $\beta$ -actin signal.

**FIGURE 4. Plasmin-cleavage attenuates PlGF-2-mediated endothelial cell chemotaxis and vascular sprouting.** (A) ConA pulldown and Western blot analysis of Nrp-1 expression in untransfected or human Nrp-1-transfected PAE cells. (B–D) Analysis of chemotactic activity of PlGF isoforms in a boyden chamber assay; PAE, PAE/Nrp-1 or HUVE cells were stimulated with (B) PlGF-isoforms (produced in Sf9 insect cells) with or without plasmin and/or  $\alpha$ 2-antiplasmin and (C, D) PlGF-isoforms (expressed in HEK293 cells); samples were analyzed in triplicates in at least 3 independent experiments, results are shown  $\pm$ SEM (n  $\geq$  3); hpf, high power field. (E) Representative images of HUVE cell spheroids embedded in collagen gels and stimulated with various PlGF isoforms (3.66 nM) (expressed in HEK293 cells), VEGF-A165 (1 nM) (positive control) or 0.1% FCS (negative control) for 24 h. (F) Quantitative analysis of cumulative sprout length/spheroid and number of sprouts/spheroid; 15 spheroids/condition were analyzed in 3 independent experiments; results are shown  $\pm$ SEM; scale bar 100  $\mu$ m.

**FIGURE 5. The HBD promotes PlGF-2-induced granulation tissue formation in diabetic mice.** (A) Representative H&E staining of PlGF-treated wound tissues of diabetic mice day 14 post injury; right panel presents boxed area in the left panel at higher magnification; dashed line outlines area of granulation tissue; e epidermis, d dermis, gt granulation tissue, sft subcutaneous fat tissue, white asterisks indicate blood vessels; scale bar 500  $\mu$ m (left panel) and 100  $\mu$ m (right panel); morphometric analysis of (B) area of granulation tissue and (C) quantification of F4/80<sup>+</sup> stained cells within a defined area (high power field, hpf) within the granulation tissue of PlGF-treated wound tissues at day 10 and day 14 post injury. Each dot represents a different wound tissue, PlGF proteins were expressed in HEK293 cells.

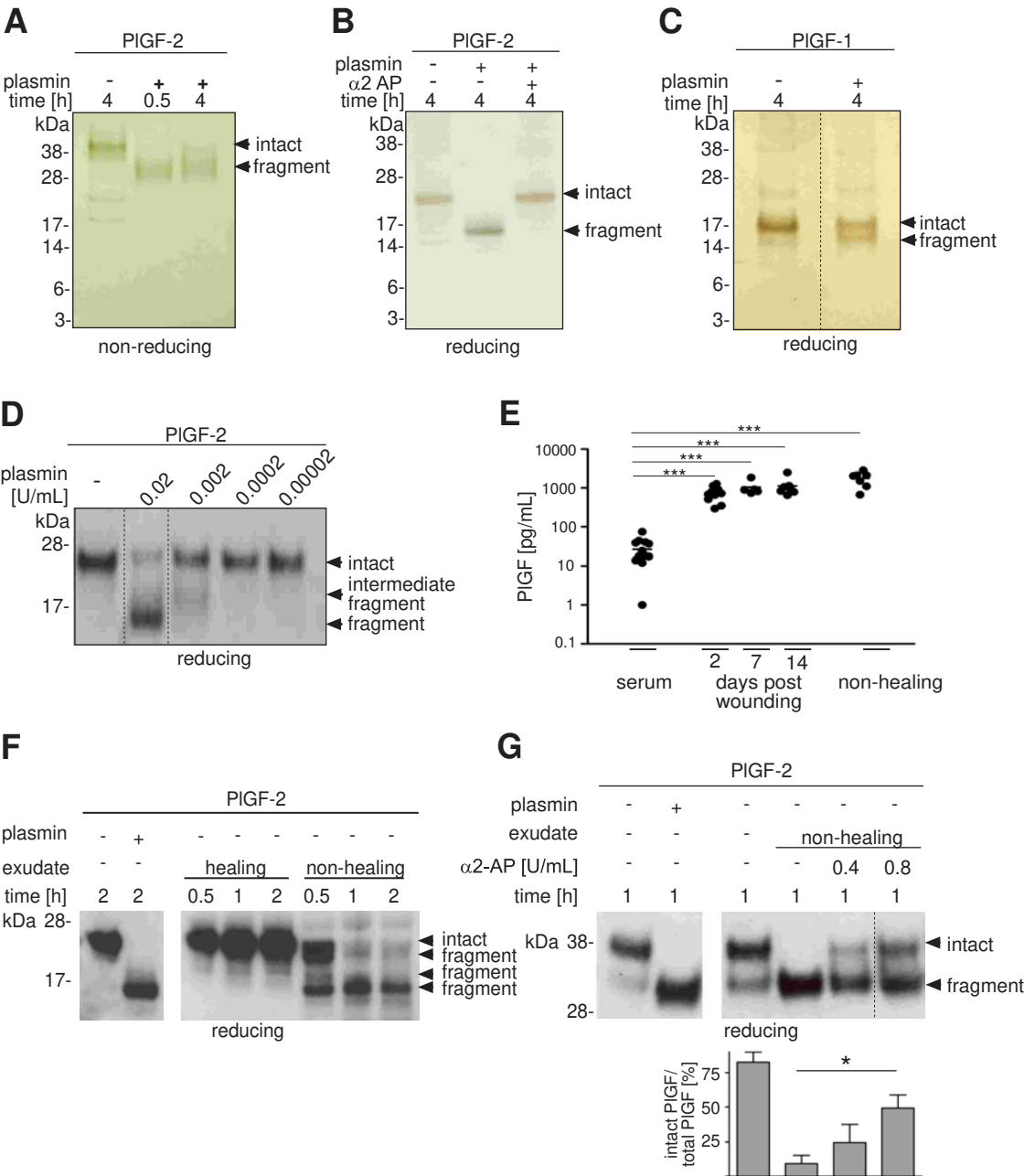
**FIGURE 6. The HBD promotes PlGF-2-induced wound angiogenesis and recruitment of perivascular cells.** (A) Representative immunohistochemical staining for endothelial cells (CD31) and perivascular cells (desmin) of PlGF-treated wound tissues in diabetic mice at day 14 post injury. (B) Quantification of the area within the granulation tissue that stained positive for CD31 or desmin at day 10 and day 14 post injury; coverage of vessels with pericytes was quantified by calculating the

ratio of desmin/CD31 double stained area; dotted line indicates epidermal-dermal junction; e, epidermis; scale bar 100  $\mu$ m, PlGF proteins were expressed in HEK293 cells.

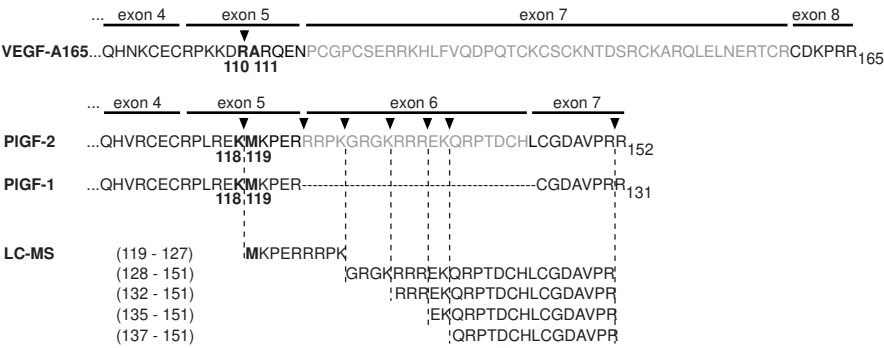
**FIGURE 7. Binding analysis of PlGF isoforms to heparin or Nrp-1.** (A) To test whether PlGF-1, PlGF-2 or PlGF118 bind to heparin, the proteins (expressed in HEK293 cells) were dialyzed against 50 mM NaCl and applied to heparin-sepharose column. Only PlGF-2 bound to heparin and was eluted with 1-2 M NaCl. (B) Nrp-1 was immobilized on sensor chips and binding sensorgrams were recorded for PlGF variants as soluble analyte; different concentrations of PlGF proteins (3-300 nM) were monitored by measuring the variation in the plasmon resonance angle as function of time and described as response units (RU). Curves are shown in ascending order depending on the analyte's concentration.

**FIGURE 8. The HBD promotes PlGF-2-induced phosphorylation of focal adhesion kinase, but is dispensable for Erk-1/-2 or Akt-mediated signaling.** Western blot analysis of signal transduction in (A) HUVE cells, (B, C) PAE/Nrp-1 or (C) PAE cells stimulated with PlGF-2, PlGF118 or VEGF-A165. Samples were subjected to SDS-PAGE and phosphorylation was analyzed by antibodies directed against phospho-Erk-1/-2 (Thr<sub>202</sub>/Tyr<sub>204</sub>), phospho-Akt (Ser<sub>473</sub>) or phospho-FAK (Tyr<sub>576</sub>/Tyr<sub>577</sub>). Antibodies directed against total proteins or  $\beta$ -actin served as loading control and were used to determine the increase over negative control. Shown are representative Western blots and the mean signal intensity  $\pm$ SEM of multiple blots ( $n \geq 3$ ); PlGF proteins were expressed in HEK293 cells. (D) Model for PlGF-mediated activities in relation to amino acid sequences encoded by exon 6 and 7. (left) PlGF-mediated phosphorylation of VEGFR-1 and downstream activation of Erk and Akt is independent of exon 6 and 7; (middle) exon 6 in PlGF-2 is essential for binding to heparin/HS or Nrp-1; (right) it is hypothesized that Nrp-1/heparin binding promotes the interaction/clustering of PlGF-2 with VEGFR-1 which then together with integrin activation leads to enhanced FAK activation and pro-angiogenic responses.

Figure 1



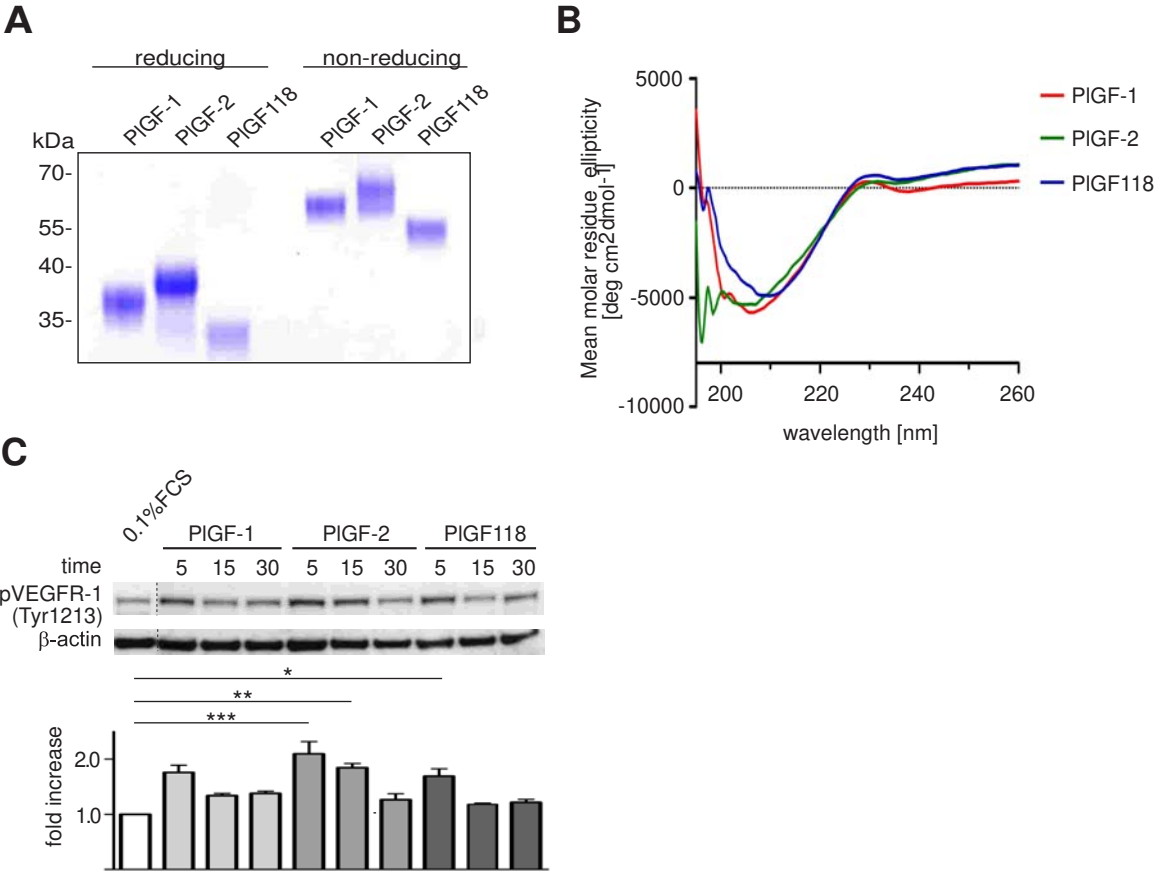
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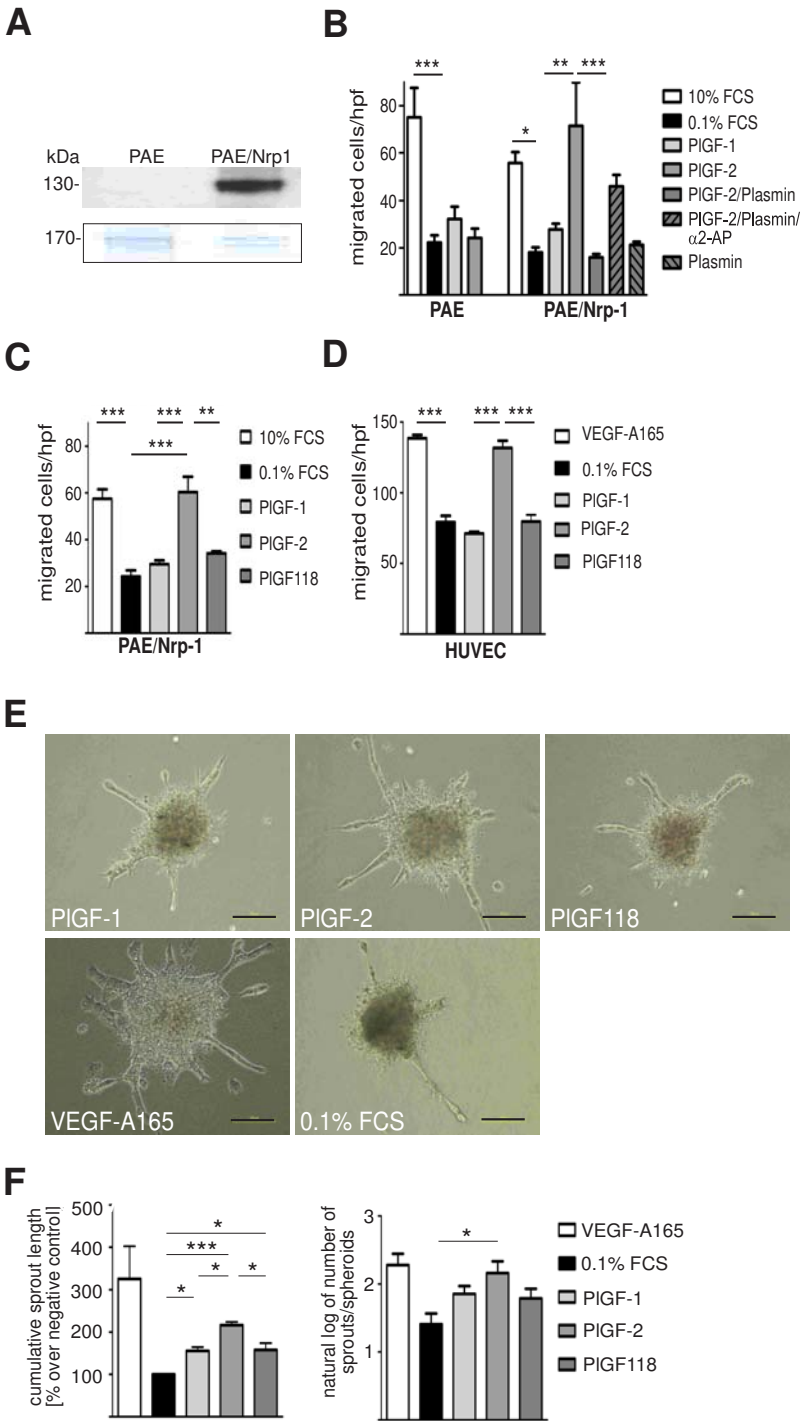


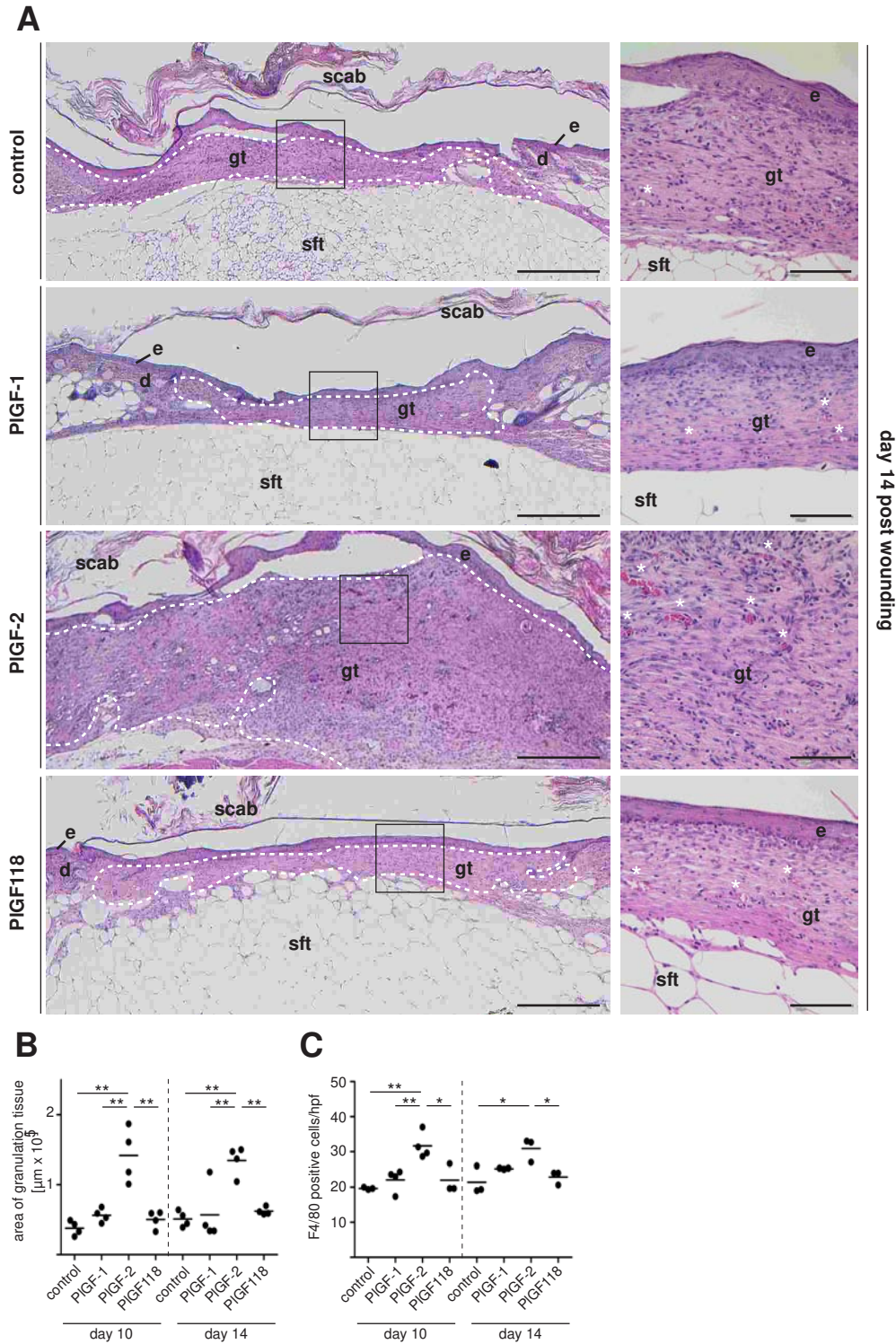
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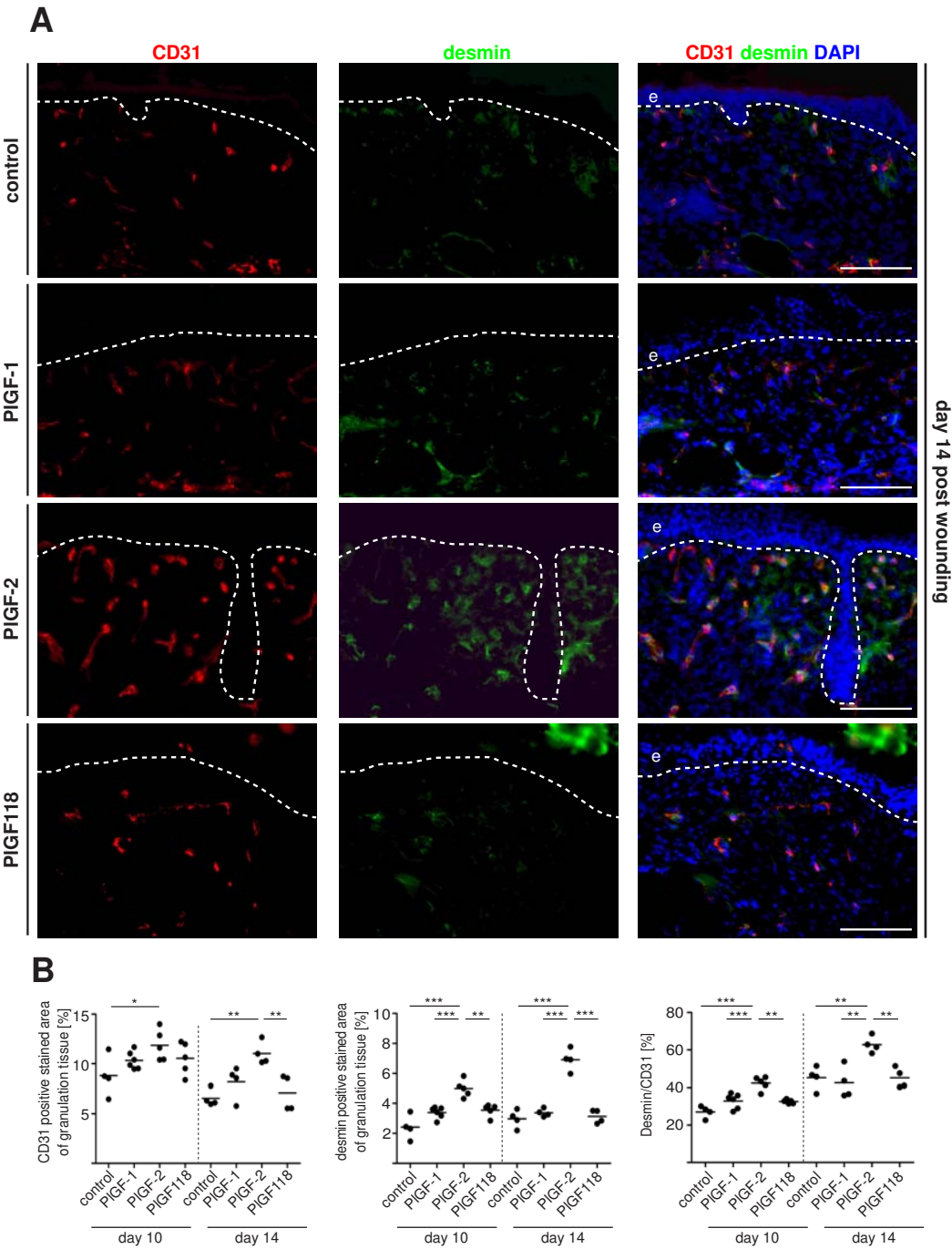
fragment	relative frequency	measured m/z [Th]	found molecular weight [Da]	expected molecular weight [Da]	absolute deviation [Da]	fragment sequence
119 - 127	+++	599.38	1196.75	1196.69	0.06	MKPERRRPK
128 - 151	+++	698.14	2788.53	2788.45	0.09	GRGKRRREKQRPTDCHLCGDAVPR
132 - 151	+++	797.77	2390.3	2390.21	0.09	RRREKQRPTDCHLCGDAVPR
135 - 151	+	641.66	1921.96	1921.9	0.06	EKQRPTDCHLCGDAVPR
137 - 151	+++	833.43	1664.84	1664.77	0.09	QRPTDCHLCGDAVPR





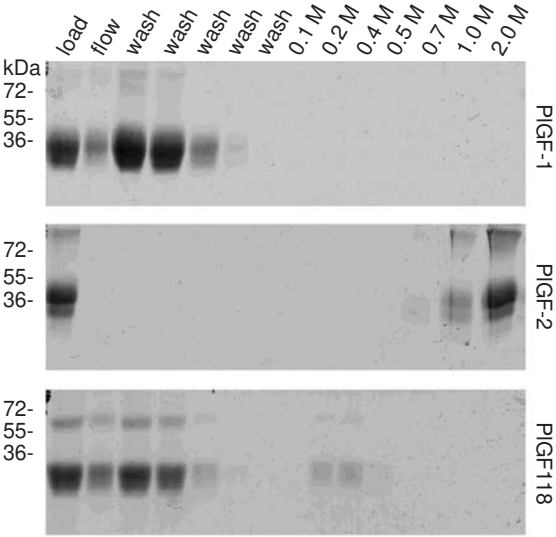








**A**



**B**

